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Sub lethal effects of ammonia at different levels of ph on chosen physiological and biochemical parameters in *Labeo rohita* (Hamilton, 1822)

L Roselin Rajathi, R Niranjani, R James
P.G. and Research Department of Zoology, V.O. Chidambaram College, Tuticorin, Tamil Nadu, India

Abstract
Effects of ammonia toxicity at different levels of pH on growth, organic reserves, respiratory enzyme activities and blood parameters were studied in *L. rohita* for 30 days. The rate and efficiency of conversion or growth were high in NH_3 + pH7 group followed by control and NH_3 + pH8 groups. However, other exposures of NH_3 + pH 6 and 9 showed a drastic and negative reduction in the rate and efficiency of conversion in *L. rohita*. The FCR value observed in NH_3 + pH7 and control groups was low as compared to other groups. The tested proximate compositions were also significantly ($P < 0.05$) reduced in fish exposed to NH_3 alone and NH_3 with acidic (pH6) and alkaline medium (pH9) as compared to other exposures. An oxidative enzyme, succinate dehydrogenase (SDH) activity and the blood parameters, RBC count, Hb count, Ht, MCV, MCH and MCHC values were high in control and NH_3 + pH7 groups and it significantly ($P<0.05$) declined in fish exposed to ammonia with increasing and decreasing of pH levels. The glycolytic anaerobic enzyme, glyceraldehyde dehydrogenase (GDH) and WBC count and ESR values showed the reverse trend against SDH activity.

Keywords: growth, proximate compositions, respiratory enzymes, ph levels, ammonia toxicity, *labeo rohita*

Introduction
Ammonia enters aquatic systems from several sources including industrial wastes, sewage effluent and agricultural input. The accumulation of ammonia in water used for intensive fish culture is a potential problem because it is toxic to fishes (Sampath, 1985; James and Sampath, 2003) [14, 12] and other aquatic species. Ammonia exists in natural water as un-ionized (NH_3) and ionized (NH_4^+) forms and among these, former is extremely toxic. Ammonia toxicity depends principally upon the presence of NH_3, which can readily diffuse across the gill membrane due to its lipid solubility and lack of charge whereas the ionized form cannot readily pass through the hydrophobic micropores in the gill membrane (Sheehan and Lewis, 1986) [31]. However, NH_4^+ is excreted across the gill only via a carrier mediated process in exchange for Na^+ and may also show considerable toxicity at low pH (Yamagata and Niwa, 1982; Chew *et al.*, 2003) [40, 4].
The acute criterion for ammonia is dependent on pH and fish species and the chronic criterion is dependent on pH and temperature (U.S.EPA, 1999). The concentration of unionized ammonia increase with increasing of pH and temperature (Randall and Tsui, 2002) [24], thereby accentuating the effects of increased unionized concentrations. The acute and chronic toxicities of ammonia have been studied on survival and growth in few freshwater fishes (Sampath *et al.*, 1991; James *et al.*, 1993; Tomasso, 1994; Metwally and Wafeek, 2014) [13, 29, 35]. However, there is paucity of information on the ammonia toxicity in relation to pH and temperature in fishes (Erickson, 1985; Mustafa *et al.*, 2006; Abbas, 2006) [7, 1, 10]. Hence, the present investigation has been undertaken to study the impacts of ammonia toxicity and pH levels on growth, organic reserves, respiratory enzymes and blood parameters in carp, *Labeo rohita.*

Materials and methods
Active and healthy juveniles (3.35±0.15g) of *L. rohita* (360 nos) were collected from acclimation tank and starved for 24 hr prior to the commencement of the experiment. They were divided into six groups of 20 individuals each in triplicates. Static renewable bio assay method was adopted (Sprague, 1973) [33] and the 96 hr $LC_{50\ value}$ was determined separately as 1.58 ppm following Finney (1971). Group 1 served as control and reared in freshwater and the pH of the medium was 7.3. Test animals belonging to 2nd to 6th groups were exposed to 0.79 mg NH_3 l^{-1} (50% sublethal level of ammonia) and pH of the media was 8.3. To assess the physiological effects of pH in contaminated medium, dilute solutions of reagent-grade hydrochloric acid or sodium hydroxide was used to maintain the test fish in ammonia media (0.79 mg NH_3 l^{-1}) with desired pH levels of 6, 7, 8 and 9 in the groups of 3, 4, 5 and 6 respectively. For convenient presentation, hereafter 3, 4, 5 and 6th groups were designated as NH_3 + pH6, NH_3 + pH7, NH_3 + pH8 and NH_3 + pH9 respectively. Before commencement of the experiment, fish were placed in the test aquaria with pH 7.3 (freshwater – control). Adjustments in pH to the desired pH levels were made gradually and monitored over 24 hr by using digital pH meter. The test media were changed daily and maintain the same ammonia level (Sprague, 1971) [33] with chosen pH levels. The experiment was conducted for 30 days in plastic troughs containing 25 *l* of water. Fish were fed with known quantum of 35% protein diet twice a day for a period of 1 hr each and thereafter unconsumed feed was removed

from the experimental troughs. After the 30 days of experiment, test animals were weighed and sacrificed for the estimations of growth, organic reserves, respiratory enzyme activities and blood parameters in *L. rohita.*

"Sacrifice method" was adopted to estimate the growth (Maynard and Loosli, 1962) [20] of the test animals. The feeding rate (mg g^{-1} live fish day^{-1}) was computed as:

$$\text{Feeding rate} = \frac{\text{Amount of feed consumed (mg)}}{\text{Initial wet weight of fish (g)} \times \text{Number of days}}$$

The growth or conversion rate (mg g^{-1} live fish day^{-1}) and gross conversion efficiency (%) were calculated as:

$$\text{Conversion rate} = \frac{\text{Growth (mg)}}{\text{Initial wet weight of fish (g)} \times \text{Number of days}}$$

$$\text{Gross Conversion Efficiency (\%)} = \frac{\text{Growth (mg)}}{\text{Feed consumed (mg)}} \times 100$$

Feed Conversion Ratio (FCR) was calculated by relating the feed consumption to gain in wet weight of fish.

$$\text{Feed Conversion Ratio} = \frac{\text{Feed consumed (g)}}{\text{Wet weight gained (g)}}$$

Organic reserves

After the experiment is terminated, few fishes were sacrificed to estimate the protein, glycogen and lipid contents and dried in hot air oven for two days. Protein was estimated by Lowry *et al*. (1951) [19], glycogen by Kemp and Kits (1954) [17] lipid by Bragdon (1951) [3] methods respectively. The enzymes, Succinate dehydrogenase (SDH) and Glyceraldehyde dehydrogenase (GDH) activities were estimated by Kun and Abood (1949) [18]. The selected biochemical parameters were also estimated in fishes prior to commencement of the experiment.

Blood Parameters

Blood parameters were estimated prior to the commencement and at the end of the experiment, Test animals were starved for 24 hr prior to the estimation of blood parameters. Three fish were removed from each experimental groups, blood was collected in a watch glass containing the required amount of 6% EDTA as an anticoagulant by cutting the caudal peduncle using a sharp knife and haematological parameters were estimated according to routine clinical methods (Wintrobe, 1978) [39]. RBC was counted using an improved Neubauer counting chamber (Feinoptik Bad Blankenburg Germany) and a haemoglobinometer (Marine field Germany) was used to determine the haemoglobin content of the blood. Red cell indices, Mean corpuscular volume (MCV), Mean corpuscular haemoglobin (MCH) and Mean corpuscular haemoglobin concentration (MCHC) were calculated from the values of haemoglobin content, Haematocrit (Ht), and the total RBC count using the formula given by Johansson – Sjobeck and Larsson (1978) [15]. The differential count of leucocytes was made by a blood smear preparation.

Statistical Analysis

Duncan multiple range test was used to determine multiple variations of mean values between control and experimental groups to assess the impacts of ammonia toxicity and pH levels in *L. rohita.*

Results

The feeding and growth parameters of *L. rohita* were high in NH_3 + pH7 group as compared to other groups. For instance, the feeding rate of NH_3 + pH7 group was 49 mg g^{-1} live fish day^{-1} and it significantly ($P < 0.05$) declined to 32, 38 and 32 mg g^{-1} live fish day^{-1} in NH_3 alone, NH_3 + pH6 and NH_3 + pH9 groups respectively. It showed that, there was no significant ($P > 0.05$) differences observed in feeding rate of NH_3 alone and NH_3 + pH9 groups (Table 1 and Fig. 1). Duncan multiple range test showed that, the rate and efficiency of conversion were high in NH_3 + pH7 group followed by control and NH_3 + pH8 groups. However, other exposures of NH_3 alone, NH_3 + pH 6 and 9 showed a drastic and negative reduction in the rate and efficiency of conversion in *L. rohita*. The FCR value observed in NH_3 + pH7 and control groups was low (3- 3.5) as compared to other groups.

The protein, glycogen and lipid contents were high in control NH_3 + pH7 and NH_3 + pH8 groups and they were drastically declined in fish exposed to NH_3 alone and fish exposed to ammonia with acidic medium (NH_3 + pH6) and alkaline medium (NH_3 + pH9). Duncan multiple range test revealed that, the tested proximate compositions were significantly ($P < 0.05$) reduced in fish exposed to NH_3 alone and NH_3 with pH6 and pH9 as compared to other exposures (Table 2). Similarly an oxidative enzyme, succinate dehydrogenase (SDH) activity was high in control and NH_3 + pH7 groups and it significantly ($P<0.05$) declined in fish exposed to ammonia with increasing and decreasing of pH levels. The glycolytic anaerobic enzyme, glyceraldehyde dehydrogenase (GDH) showed the reverse trend against SDH activity (Table 2).

Like growth and enzymes activities, RBC count, Hb content, Ht, MCV, MCH and MCHC values were high in control and NH_3 + pH7 groups and they were significantly ($P < 0.05$) declined in fish exposed to NH_3 alone and NH_3 with pH6 or pH9. However, an opposite trend was observed in WBC count and ESR values was observed in ammonia exposed fish at pH9, followed by pH6 and NH_3 alone while other exposures exhibited the low values. Duncan multiple range test revealed that, fish exposed to acidic or alkaline medium significantly ($P < 0.05$) reduced the RBC count, Hb content, Ht and other values while WBC count and ESR values were tremendously increased as compared to other exposures (Table 3). The percentage of lymphocytes and neutrophil were drastically increased in ammonia exposed fish with acidic (pH6) and alkaline (pH9) media as compared to other media. However, the populations of basophil and thrombocyte showed the opposite trend (Table 4).

Table 1: Sublethal effects of ammonia on food utilization parameters in *Labeorohita*as a function of pH levels. Each value is the mean $(\bar{X} \pm SD)$ of three observations. Rates are expressed as mg g^{-1} live fish day^{-1} and efficiencies as percent.

Parameters	C	NH_3 alone	NH_3 + pH6	NH_3 + pH7	NH_3 + pH8	NH_3 + pH9
Initial MBW (g wet wt.)	^{a}15.58±0.40	^{a}15.53±0.25	^{a}15.71±0.88	^{a}15.92±0.73	^{a}15.43±0.08	^{a}15.52±0.41
Final MBW (g wet wt.)	^{d}21.38 ± 0.38	^{a}12.82±0.34	^{b}14.41±0.53	^{e}23.04±0.45	^{c}20.15±0.19	^{a}12.05±0.34
Weight gain (g wet wt.)	^{e}5.80 ± 0.04	^{b}-2.71±0.01	^{c}-1.30 ± 0.03	^{f}7.12 ± 0.17	^{d}4.72 ± 0.34	^{a}-3.47 ± 0.27
Feed intake (g wet wt.)	^{c}20.18 ± 1.09	^{a}15.08±1.13	^{b}17.90±0.98	^{c}22.13±0.85	^{c}19.70±1.01	^{a}14.83±0.53
Feeding rate	^{c}43.18± 0.55	^{a}32.37±0.97	^{b}37.98±0.39	^{d}49.17±0.90	^{c}42.55±0.35	^{a}31.84±0.52
Conversion rate	^{e}12.40 ± 0.07	^{b}-5.82±0.01	^{c}-2.73 ± 0.05	^{f}14.91±0.53	^{d}10.19±0.91	^{a}-7.45 ± 0.09
Conversion efficiency	^{e}28.74 ± 1.74	^{b}-17.97± 0.05	^{c}-7.23±0.01	^{f}33.17±1.04	^{d}23.95±1.93	^{a}-23.39 ±0.53
Feed conversion ratio (FCR)	^{d}3.47 ± 0.29	^{b}-5.57±0.43	^{a}-13.77± 0.05	^{d}3.10±0.29	^{e}4.17±0.12	^{c}-4.27±0.07

Values (mean ±SD) with different superscript in the same row are significantly different (P<0.05)

Table 2: Sublethal effects of ammonia on proximate compositions (mg g^{-1} dry matter) and dehydrogenase enzyme activities of succinate and glyceraldehyde (µg reduced TTC/100 mg dry matter) in *Labeorohita*as a function of pH levels. Each value is the mean $(\bar{X} \pm SD)$ of three estimations.

Parameters	Initial	Control	NH_3 alone	NH_3 +pH6	NH_3 + pH7	NH_3 + pH8	NH_3 + pH9
Protein	14.57±0.27	^{c}17.23 ± 0.54	^{b}7.05 ± 0.34	^{b}6.71±0.59	^{d}18.09±0.34	^{c}17.09 ± 0.25	^{a}5.45 ± 0.39
Glycogen	5.73 ± 0.23	^{d}8.73 ± 0.35	^{a}1.78 ± 0.40	^{a}1.55±0.21	^{c}7.38 ± 0.23	^{b}6.72 ± 0.54	^{a}1.35 ± 0.10
Lipid	1.15 ± 0.47	^{b}1.70 ± 0.34	^{a}0.19 ± 0.08	^{a}0.25±0.13	^{b}1.73 ± 0.10	^{b}1.55 ± 0.11	^{a}0.07 ± 0.002
Enzyme activities							
SDH	10.98±0.17	^{c}12.07 ± 0.87	^{b}5.80 ± 0.05	^{b}5.94±0.21	^{d}14.95±0.59	^{c}11.95 ± 0.71	^{a}4.73 ± 0.11
GDH	23.75±2.01	^{a}21.44 ± 1.45	^{d}53.04±2.48	^{c}40.12±2.55	^{a}23.93±2.03	^{b}29.05±3.11	^{e}57.83 ± 0.71

Values (mean ±SD) with different superscript in the same row are significantly different (P<0.05)

Table 3: Sublethal effects of ammonia on blood parameters in *Labeorohita*as a function of pH levels. Each value is the mean $(\bar{X} \pm SD)$ of three estimations**.**

Parameters	Initial	Control	NH_3 alone	NH_3 + pH6	NH_3 + pH7	NH_3 + pH8	NH_3 + pH9
RBC($\times 10^6$ mm^{-3})	2.17 ± 0.21	^{b}2.55 ± 0.10	^{a}0.93 ± 0.81	^{a}0.98±0.13	^{b}2.43 ± 0.54	^{b}2.05 ± 0.19	^{a}0.75 ± 0.23
Hb(g%)	2.89 ± 0.07	^{f}4.35 ± 0.14	^{c}1.03 ± 0.03	^{b}0.84±0.01	^{e}2.90 ± 0.05	^{d}2.29 ± 0.11	^{a}0.58 ± 0.03
WBC ($\times 10^3$ mm^{-3})	31.13± 1.13	^{a}28.79 ± 0.73	^{c}45.12±0.95	^{d}47.85±0.89	^{a}30.19 ± 0.93	^{b}39.93±0.32	^{e}53.79±1.07
ESR (mm^{-1}hr)	3.95±0.33	^{a}2.98±0.18	^{c}5.13±0.17	^{d}5.98±0.23	^{b}3.98 ± 0.21	^{b}4.29 ± 0.19	^{e}7.13 ± 0.25
Haematocrit (%)	23.33±0.48	^{f}29.98 ± 0.13	^{c}9.05 ± 0.23	^{b}7.83±0.44	^{e}21.93 ±0.73	^{d}19.58±0.39	^{a}5.89 ± 0.12
MCV(fl)	107.35±4.89	^{d}117.57 ± 5.93	^{c}97.31±3.43	^{a}79.89±3.39	^{b}90.23±7.31	^{b}95.52±4.58	^{a}78.53 ± 2.53
MCH(pg)	13.31±1.03	^{d}17.05 ± 1.84	^{b}11.08±1.11	^{a}8.57±0.53	^{c}15.44 ± 0.38	^{c}14.14 ± 0.45	^{a}7.73 ± 1.01
MCHC(g^{-1})	12.38± 0.13	^{b}14.50 ± 1.28	^{a}11.39±0.53	^{a}10.72±1.78	^{b}15.01±0.33	^{b}14.81±0.49	^{a}9.85 ± 0.31

Values (mean ±SD) with different superscript in the same row are significantly different (P<0.05)

Table 4: Sublethal effects of ammonia on differential leucocyte count (%) in *Labeorohita*as a function of pH levels. Each value is the mean $(\bar{X} \pm SD)$ of three estimations.

Components	Initial	Control	NH_3 alone	NH_3 + pH6	NH_3 + pH7	NH_3 + pH8	NH_3 + pH9
Neutrophil	13.01± 0.21	^{ab}12.34 ± 0.17	^{e}17.98±0.10	^{c}15.83±0.35	^{a}12.14±0.45	^{b}12.85±0.11	^{d}17.17±0.39
Basophil	7.18 ± 0.17	^{b}7.05 ± 0.03	^{d}9.18 ± 0.19	^{c}8.39±0.45	^{a}5.31 ± 0.25	^{b}7.11 ± 0.23	^{d}9.31 ± 0.30
Thrombocyte	45.57±2.31	^{f}49.85 ± 2.48	^{b}25.72±2.18	^{c}29.85±2.19	^{e}43.11±2.25	^{d}37.18±2.17	^{a}21.13 ± 2.53
Lymphocyte	34.24±1.10	^{a}30.75±2.95	^{c}47.12±2.38	^{c}45.93±3.10	^{b}38.91±2.28	^{c}43.57 ± 2.85	^{d}52.39±0.93

Values (mean ±SD) with different superscript in the same row are significantly different (P<0.05)

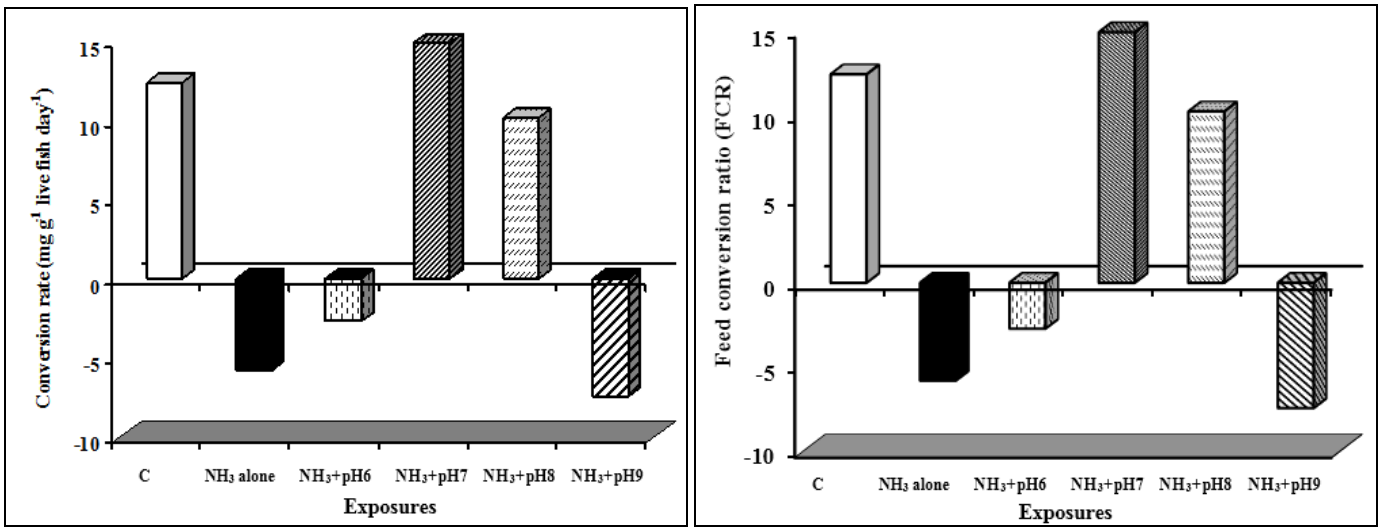

Fig 1: Sublethal effects of ammonia on conversion rate and feed conversion ratio in *Labeo rohita* as a function of pH levels.

Discussion

The present study revealed that, growth parameters were drastically declined in *L. rohita* exposed to sublethal level of ammonia alone and ammonia with acidic medium (pH6) and alkaline medium (pH9). The reduction of growth rate and efficiency may be due to the unionized form of ammonia toxicity as it passes readily through the biological membrane (Hampson, 1976) [9]; the proportions of this form of ammonia influenced by temperature, pH and ionic concentrations of water (James *et al.,* 1993) [13]. Ammonia levels in culturable medium causes the physiological stress responses, leading to decreased growth and survival of the fish (Wedemeyer and McLeay, 1981; Tomasso, 1994) [35, 37]. Willingham *et al.* (2004) [38] attributed that, feed consumption was high in fish reared with nitrogenous wastes at pH 7.2 – 7.9 and thereafter, it declined as the level of pH increased supports the present observation. Sherif and Feky (2009) [6] reported that, growth rate of Nile tilapia (*Oreochromis niloticus*) fingerlings was high when it exposed to the level of pH7 followed by pH8, pH9 (alkaline medium) and pH6 (acidic medium) which strongly supports the present investigation. Also Saha *et al.* (2002) [26] and Scott *et al.* (2005) [30] found that, ammonia toxicity increased with increasing of pH (alkalinity) levels resulted the decline of the growth rate in fishes due to the reduction in feed intake.

The significant ($P < 0.05$) reduction of protein, glycogen and lipid contents were observed in *L. rohita* exposed to sublethal level of ammonia alone and ammonia with acidic (pH6) or alkaline medium (pH9). It indicates the utilization of more organic reserves during the stress caused by ammonia toxicity and it also enhanced by both acidic (pH6) and alkaline (pH9) media. Working on *C. carpio* fingerlings, Abbas (2006) [10] found that, decline of glucose in ammonia exposed fish at different pH levels. A similar effect was measured in rainbow trout *Onchorhynchus mykiss* exposed to 0.34 mg NH_3 l^{-1}(Thurston *et al.,* 1978) and in grass carp, *Ctenopharyngodon idella* exposed to 0.7 mg NH_3 l^{-1} at pH 8.5 supports the present study (Salah EL-Deen, 1999) [27].

The suppression of SDH activity in NH_3 + pH6 or pH9 groups indicates an impairment of oxidative metabolic cycle and hence depend on anaerobic glycolysis to meet energy demands as evident by the elevation in GDH activity. This suggests a metabolic shift from aerobiosis to anaerobiosis. The metabolic shift appears as an adaptation for survival under chronic ammonia stress and the stress was enhanced to many fold with increasing of pH levels (pH8 - pH9) and at low pH6. Metwally and Wafeek (2014) [21] reported that, an elevation of dehydrogenase activities of lactate (LDH) and glucose 6 – phosphate (G6PDH) in ammonia exposed Nile tilapia, *O. niloticus* supports the increasing activity of GDH in *L. rohita* exposed to NH_3 + pH levels.

In the present study, the decline of RBC count, Hb content and Ht value were observed in NH_3 + pH6 and NH_3 + pH9 groups, could be attributed to shrinkage of erythrocytes, decline of erythrocytes production in the hematopoietic tissue and hemodilution and intravascular destruction due to the combined stress and toxicity of ammonia and pH. Similar finding was reported in *C. carpio* (Abbas, 2006) [10], *O. niloticus* (Ahmed *et al.,* 1992) [2] and *C. idella* (Salah EL-Deen, 1999) [27] exposed to toxic concentrations of ammonia. An increase of WBC count and ESR value in *L. rohita* exposed to sublethal level of ammonia alone and NH_3 + pH9 or pH6, suggests the triggering of cell defense mechanism against the stress caused by ammonia and pH. Increase of WBC count in NH_3 + pH6 or pH9 might be due to the increase in the population of lymphocytes, neutrophils and basophils (Table 7.5). The increase in lymphocytes suggests that, the immune mechanism of fish gets stimulated and becomes adapted under ammonia stress to fight against the toxicants in the environments (James and Sampath, 1996) [11]. The neutrophil increase in above exposures, seems to be caused by tissue damage. Sampath *et al.* (2003) [14] reported that neutrophils were the most important of the leucocytes as they show greatest sensitivity to change in the environment. Similar elevation of neutrophil was also observed in *C. carpio* exposed to copper with different levels of pH (Mottahari *et al.,* 2013) [25].

The present study concludes that, ammonia toxicity is strongly correlated with fluctuations in the pH of the system (Randall and Tsui, 2002; Mustafa *et al.*, 2006) [24, 1]. It suggests that

careful regulation of pH and ammonia can lead to avoidance of excessive stress and better growth and survival of farmed fish.

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